

Tendon cells in vivo form a three dimensional network of cell processes linked by gap junctions

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ABSTRACT

Tendons respond to mechanical load by modifying their extracellular matrix. The cells therefore sense mechanical load and coordinate an appropriate response to it. We show that tendon cells have the potential to communicate with one another via cell processes and gap junctions and thus could use direct cell/cell communication to detect and/or coordinate their load responses. Unfixed cryosections of adult rat digital flexor tendons were stained with the fluorescent membrane dye DiI to demonstrate cell shape. Similar sections were immunolabelled with monoclonal antibodies to rat connexin 32 or connexin 43 to demonstrate gap junctions and counterstained with propidium iodide to show nuclei, or the membrane stain DiOC₇ to show cell membranes. Sections were examined with a laser scanning confocal microscope and 3-dimensional reconstructions were prepared from optical section series to demonstrate cell shape and the position of connexin immunolabel. Cells had a complex interconnected morphology with gap junctions at points of contact with other cells. Cell bodies contained the nucleus and extended broad flat lateral cell processes that enclosed collagen bundles and interacted with similar processes from adjacent cells. They also had long thin longitudinal processes interacting with the cell process network further along the tendon. Connexin 43 occurred where cell processes met and between cell bodies, whereas connexin 32 was only found between cell bodies. The results indicate the presence of a 3-dimensional communicating network of cell processes within tendons. The intimate relationship between cell processes and collagen fibril bundles suggests that the cell process network could be involved in load sensing and coordination of response to load. The presence of 2 different types of connexins suggests that there could be at least 2 distinct communicating networks.

Key words: Tendon; cell shape; connexins; cell–cell communication.

INTRODUCTION

Tendons, which transfer tensile loads from muscles to bone, consist of tendon fibroblasts arranged in short longitudinal rows separated by longitudinally running bundles of collagen fibrils (Benjamin & Ralphs, 1995). They are often regarded as inert ‘ropes’ but in fact exhibit considerable dynamic behaviour in altering the composition of their extracellular matrix (ECM) in response to mechanical loads, a common feature of connective tissues. Tendon cells that experience compressive as well as tensile loads, in vivo or in vitro, synthesise ECM more typical of cartilage, forming fibrocartilage to resist the compression; in the absence of compressive forces, they revert to pure tendon (e.g.

Vogel & Koob, 1989, Benjamin & Ralphs, 1995). Immobilisation of tendons, experimentally or as part of treatment for other injuries, leads to depletion of the ECM and substantial weakening; remobilisation eventually results in the restoration of strength (Amiel et al. 1995). The treatment of choice for traumatic or surgical injury is continuous passive motion which substantially accelerates healing (Buckwalter, 1996). Tendon cells thus detect the loads they experience and respond appropriately to them.

Cellular mechanisms for sensing load have not been elucidated, although the most commonly postulated systems involve ECM receptors, integrins, linking the ECM to the cytoskeleton within the cell (see Benjamin et al. 1994 for review). There is also evidence for a

mechano-electrical sensing system, where mechanically sensitive ion channels alter membrane permeability under load. If tendon cells are deformed *in vitro*, by indenting the plasma membrane, there is a rapid internal calcium transient which is propagated to adjacent cells by the transfer of inositol triphosphate (IP^3) via gap junctions (Banes et al. 1995). Thus signals derived from mechanical stimuli can be transferred cell to cell and could mediate and coordinate cell responses. Tendon cells express the gap junction protein connexin (cxn) 43 *in vitro*, alter its phosphorylation in the presence of growth factors (Banes et al. 1995; phosphorylation state probably relates to the ability of junctions to pass ions) and upregulate its expression in response to load (Banes et al. 1996). How these results relate to the *in vivo* behaviour of tendon cells is not known, although there is an isolated report of gap junctions being observed ultrastructurally between tendon cells at the myotendinous junction (Tangi et al. 1995); in the dermis (another dense fibrous connective tissue) dye transfer has been demonstrated between fibroblasts in tissue samples (Pitts et al. 1987).

For gap junctional communication to be important in tendon cell behaviour *in vivo*, cells must have direct membrane-membrane interaction, and the membranes must contain gap junction proteins. There is some evidence that direct cell-cell interaction is possible in tendons: clearly cells within longitudinal cell rows are in contact with other cells in the same row. There is also evidence of lateral cell-cell interaction in the deposition of oriented bundles of collagen fibrils in development, where macro-aggregates of collagen bundles are enclosed by cell processes from 2–3 adjacent fibroblasts (Birk & Zycband, 1994); lateral cell/cell interactions have also been described ultrastructurally in adult tendons (Merrilees & Flint, 1980; Squier & Magnes, 1983; Senga et al. 1995). However, the significance of such cell-cell contact has not been investigated in a wider context. In the present study, we have used whole cell fluorescent labelling and immunocytochemistry, allied to confocal laser scanning microscopy and computer modelling, to demonstrate that there is indeed an elaborate interconnecting network of cell processes in rat tendons, and at least 2 different types of gap junctions on the interacting plasma membranes.

MATERIALS AND METHODS

Material

Deep flexor tendons were dissected from the hind feet of sexually mature (3–4-mo-old; $n = 20$) male and

female white Wistar rats. Specimens were rinsed in phosphate buffered saline (PBS), fresh frozen onto cryostat stubs using dry ice, and 20 μ m cryosections cut in longitudinal or transverse planes.

Cell staining with DiI

To examine the 3-dimensional shape of tendon cells, unfixed cryosections were incubated for 10 min with the membrane label DiI (10 μ M in PBS; Molecular Probes). Sections were then examined using a Molecular Dynamics Sarastro 2000 confocal laser scanning microscope (CLSM) set up for rhodamine illumination and detection (laser wavelength 514 nm, beamsplitter 535 nm, barrier filter 535 nm). Using $\times 40$ and $\times 60$ objectives, optical section series were taken through the whole cryosection thickness and 3D projections prepared using Molecular Dynamics ImageSpace software running on a Silicon Graphics XS24 4000 workstation.

Immunolabelling and counterstaining

To investigate the distribution of gap junction proteins within tendons, cryosections were immunolabelled with antibodies to connexins and counterstained with fluorescent dyes to see the immunolabel in context. Monoclonal anti-cxn43 (Zymed or Chemicon International Ltd) was raised to a synthetic peptide corresponding to positions 252–270 of native cxn43 (Beyer et al. 1988). Monoclonal anti-rat cxn32 (Chemicon International Ltd) recognises an epitope located between residues 95 and 125 in the central cytoplasmic loop (Goodenough et al. 1988). Immunolocalisations were performed using standard techniques for indirect immunofluorescence, with anti-cxn43 being used at 2 μ g/ml and anti-cxn 32 at 10 μ g/ml. Second antibodies were sheep antimouse IgG Fab fragments/FITC conjugate (1:100; Sigma Chemical Co.) or rabbit antimouse IgG conjugated with the red fluorogen CY3 (1:400; Chemicon International Ltd) according to the fluorescence colour of the counterstain. Control sections were incubated with nonimmune mouse immunoglobulins (10 μ g/ml) or the primary antibody was omitted. The counterstains were propidium iodide, to make nuclei fluoresce red (0.5 μ g/ml; Molecular Probes) and the cyanin dye DiOC₇ (8 μ M; Molecular Probes) which stains membranes fluorescent green. For propidium iodide labelling, sections were postfixated in 90% methanol at 4 °C for 2 min before immunolabelling, and then treated with propidium iodide for 5 min at

the end of the procedure. For DiOC₇, sections were left unfixed and incubated with the counterstain for 30 min at the end of the immunolabel procedure. All sections were mounted in an aqueous mountant containing diazobicyclo 2,2,2 octane as fluorescence preservative (Johnstone et al. 1982). For all counterstaining procedures, the CLSM was set up for dual channel fluorescence using fluorescein and rhodamine filter sets (laser wavelength 514 nm, primary beam-splitter 535 nm, secondary beamsplitter 565 nm, detector filters 570 nm and 540 nm). Section series were taken and 3D modelling performed as above. For easy comparison between DiOC₇/CY3 and propidium iodide/FITC labelled specimens, the red/green colours of the former were reversed during computer modelling.

Whole tendon labelling

2–3 mm lengths of freshly dissected unfixed tendons were soaked in DiI, as used above, for 1 h, rinsed thoroughly in PBS and mounted under a supported coverslip so that there was only a slight flattening of the tendon. Using the rhodamine filter set, laser power set to maximum (25 mW) with no laser attenuation and detectors set to high sensitivity, it was possible to obtain images of fluorescing cells up to approximately 90 µm beneath the surface of the tendon. 3D models were made of section series taken using the $\times 40$ objective, as above.

In addition, whole tendon immunolabelling was performed. 2–3 mm pieces of tendon were fixed for 30 min in 90% methanol at 4 °C, washed, incubated in monoclonal anti-cxn43 for 24 h, rinsed for 12 h in several changes of PBS and then incubated with the FITC antimouse Fab fragments conjugate for a further 24 h. The specimens were again extensively washed, and mounted as above. The CLSM was set up at similar high power settings to those outlined above, but in this case using the fluorescein filter set.

RESULTS

Cell shape and organisation

At low magnification, transverse sections stained with DiI showed that the flexor tendons were quite cellular, with flattened epitenon cells surrounding the tendon proper (Fig. 1*A*). Even at this low magnification interconnections between cells could be observed, and higher magnification revealed remarkable complexities in the shape and organisation of tendon cells and in their association with collagen bundles (Fig.

1*B*). Tendon cells had many cell processes extending into the ECM. In 3D models created from transverse section series, cells could be seen to extend lateral sheet-like cell processes which wrapped around collagen bundles and contacted similar processes from laterally adjacent cells. In models created from longitudinal section series (Fig. 1*C*), cells arranged in short longitudinal rows were clearly in contact with one another, with those at the end of rows extending long thin cell processes longitudinally through the tendon matrix. These connected with the network of cell process of other groups of cells along the tendon. To generalise these observations, a cell in the mid-part of a cell row had a relatively short cell body, contacting its neighbours anteriorly and posteriorly, and extensive lateral sheet-like cell processes which contacted similar processes extending from cells in laterally adjacent cell rows. Cells at the ends of rows had their lateral cell sheets but also long thin longitudinal cell processes. There was thus an extensive network of cell processes running throughout the ECM. Optical sectioning of DiI stained tendon whole mounts 30–40 µm below the surface of the tendon showed rows of tendon cells associated with collagen fibril bundles and demonstrated that a given fibril bundle was intimately associated with successive tendon cells of a row (Fig. 1*D*).

Epitenon

At the tendon periphery there were 2–3 layers of flattened cells forming the epitenon. In glancing optical sections of the periphery, the outermost layer appeared to form a complete cellular sheet covering the tendon (Fig. 1*E*). In reconstructions made from transverse sections the innermost layer had many cell processes linking with similar processes from the first region of cells of the tendon mid-substance (Fig. 1*F*).

Distribution of CXN 43 and 32

All cell processes had bright foci of immunolabel for cxn43 wherever they made contact with another cell, indicating the presence of gap junctions (Fig. 2*A–D*). The foci were clearly visible between lateral cell processes (Fig. 2*A*), between cell bodies and between longitudinal cell processes (Fig. 2*B*). Connexin 43 was particularly prominent at the periphery of the tendon in the region rich in cell processes between the innermost epitenon cell and the outermost tendon cell proper (Fig. 2*C*). Optical sections through whole mount immunolabels clearly demonstrated the large

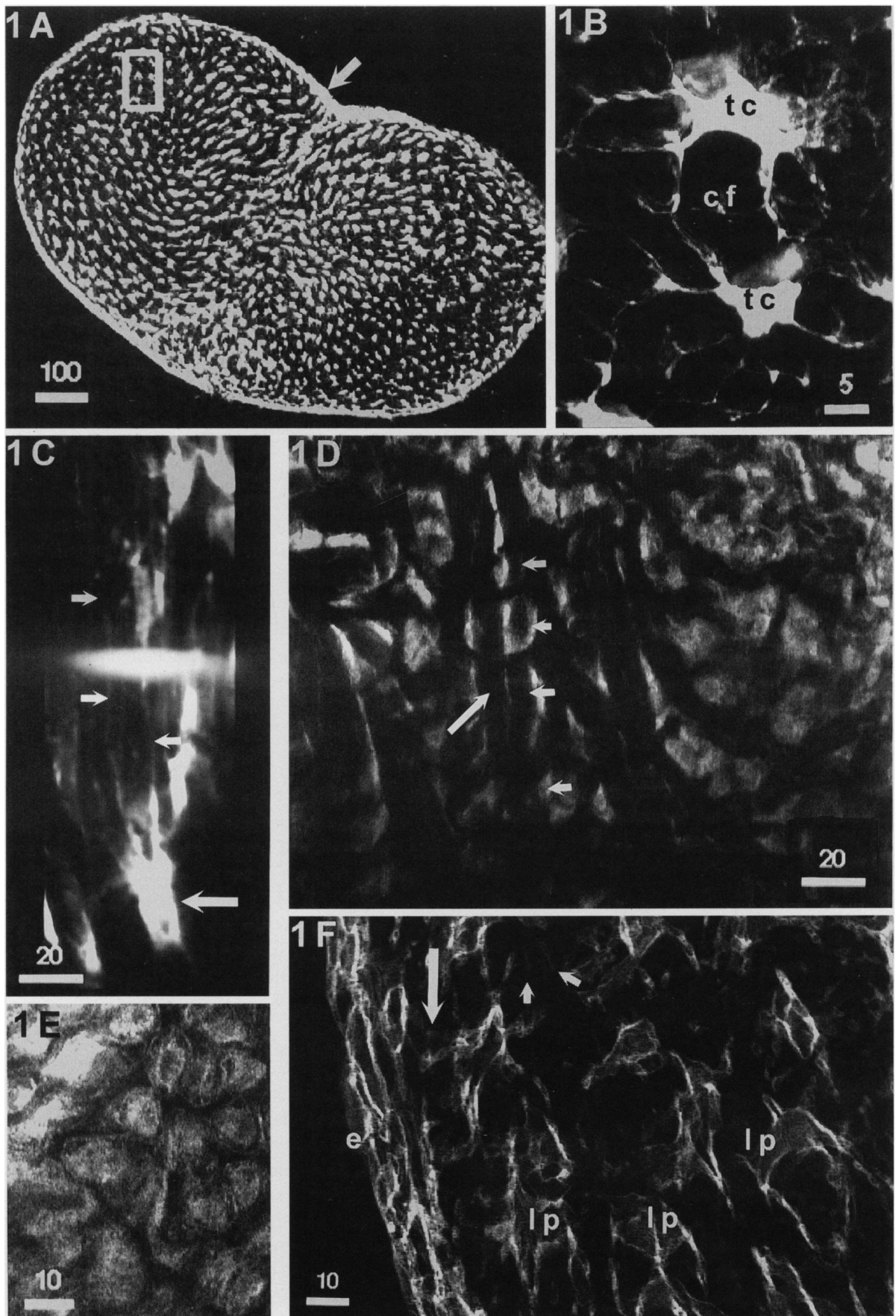


Fig. 1. For legend see opposite.

number of gap junctions within the tendon, often showing ordered linear arrays, presumably where two or more lateral cell processes met (Fig. 2D).

Label for cxn32 also appeared to be more prominent towards the periphery of the tendon, but when cells were examined in detail it had a rather different cellular distribution (Fig. 2E,F). Cxn32 immunolabel was more diffuse on cells than cxn43, with many small individual foci of label between cells resolvable in high magnification projections (Fig. 2F). At low magnification the foci were not resolvable and cells appeared to have large plaques of label on their surfaces. Generally cxn32 was associated with junctions between cell bodies rather than cell processes; cxn43 was always associated with cell processes but cxn32 was rarely observed at this site (Fig. 3A,B).

DISCUSSION

This study demonstrates that tendon cells are complex in shape, forming a 3-dimensional network of cell processes associated with collagen bundles and distributed throughout the tendon. Cells in rows have flattened cell processes extending laterally and connecting with those from laterally adjacent cells. Cells at the ends of rows have the lateral processes and also long thin longitudinal processes which make contact with cell processes or cell bodies of cells some distance away longitudinally. At the periphery of the tendon, flattened epitenon cells form a complete pavement of cells over the tendon surface and from their internal surface have many cell processes contacting the first peripheral tendon cells proper. Thus, with the presence of gap junctions between cell processes, there is the potential for a tendon-wide communicating cellular network, similar to that seen between osteocytes in bone (e.g. Jones et al. 1993; Lanyon, 1993).

The shape of tendon cells has been described at ultrastructural level in the context of the laying down of collagen fibrils by cells during tendon development. Cells use a hierarchy of 3 extracellular compartments

to lay down their oriented collagen matrix (Birk & Zycband, 1994). First, 1–3 fibrils form in narrow channels linked to the cell surface that originate deep in the cytoplasm. Secondly, these channels fuse together to form bundles of collagen fibrils in close association with the cell surface. Thirdly, bundles become laterally associated to form macroaggregates, in a compartment defined by the apposition of 2–3 fibroblasts. Any given fibroblast is associated with several fibril bundles. We clearly see these 3rd compartments with our membrane stains of cryosections. In addition, we can define a 4th compartment. The whole mount studies clearly demonstrate that fibril bundles, contained in the 3rd compartment delineated by laterally adjacent cells, pass from cell to cell longitudinally in the tendon so that each fibril bundle is completely enclosed in wrappings of the fine sheet-like lateral cell processes. The 4th compartment, therefore, is that defined by longitudinal rows of cells associated with the same fibril bundle. The gap junction results suggest that laterally and longitudinally adjacent cells can co-operate with one another in the laying down and maintenance of fibril bundles. The presence of gap junctions between tendon cells demonstrates the possibility of direct cell–cell communication between tendon cells throughout the tendon. Expression of cxn43 and 32 in vitro correlates with direct cell communication, as shown by dye coupling and electrical conductivity; however cxn 43 and 32 containing gap junctions can have very different dye coupling and electrical characteristics (e.g. Veenstra et al. 1992; Elfgang et al. 1995). This, taken with our observation of different cellular distributions of the 2 connexins, suggests tendon cells have 2 distinct populations of gap junctions on their surfaces. It may be that the 2 types of junction mediate different activities within the tendon cell network.

Since gap junctions couple cells electrically and chemically, they could coordinate response to mechanical load, detect damage or allow metabolic co-

Fig. 1 A–F. Confocal microscope images of DiI stained cryosections and wholemounts of rat digital flexor tendons. Scale bars are labelled in μm . (A) Low magnification of transverse cryosection of whole tendon. Cells are brightly fluorescent; close examination reveals a fine network of fluorescent cell processes between cells (boxed area enlarged in B). The whole tendon is enclosed by 2–3 layers of flattened cells, the epitenon (arrow). (B) High magnification 3D projection (look-through reconstruction) of boxed region in A. Tendon cells (tc) extend broad flattened lateral cell processes, meeting up with those from adjacent cells. The processes wrap up collagen fibre bundles, occupying the dark tunnels (cf). (C) 3D projection (look-through reconstruction) of a tendon cell in longitudinal cryosection. This cell is at the end of a cell row and has long fine cell processes that extend longitudinally along the tendon (small arrows) from the cell body (large arrow). (D) Projection made from optical sections 30–40 μm below the surface of a tendon whole mount. Dark ‘tunnels’ containing collagen bundles (large arrows) pass from cell to cell (small arrows). (E) Tangential optical section at the surface of a tendon whole mount showing the flattened sheet of epitenon cells. (F) 3D projection (look-through, surface extraction reconstruction) of the periphery of a transverse cryosection. This reconstruction shows the layers of cells in the epitenon (e) and demonstrates the large number of cell processes within the tendon – there are many processes between the epitenon cells and the tendon cells themselves (arrow). Flattened sheets and tubes of membrane are visible deeper in the tendon where lateral cell processes are wrapped around collagen bundles (lp). Some fine longitudinal processes can be seen passing through the thickness of the cryosection (small arrows).

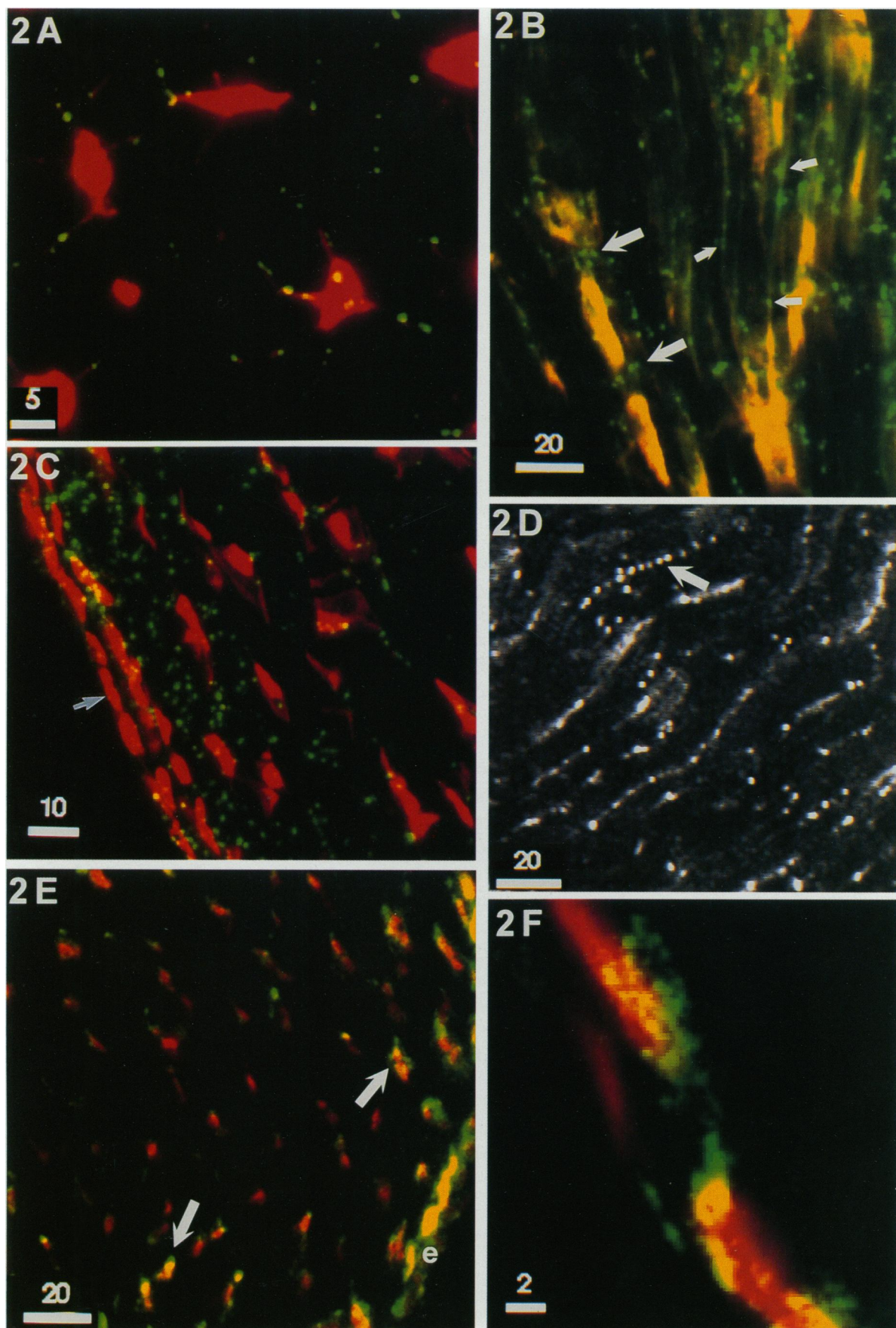


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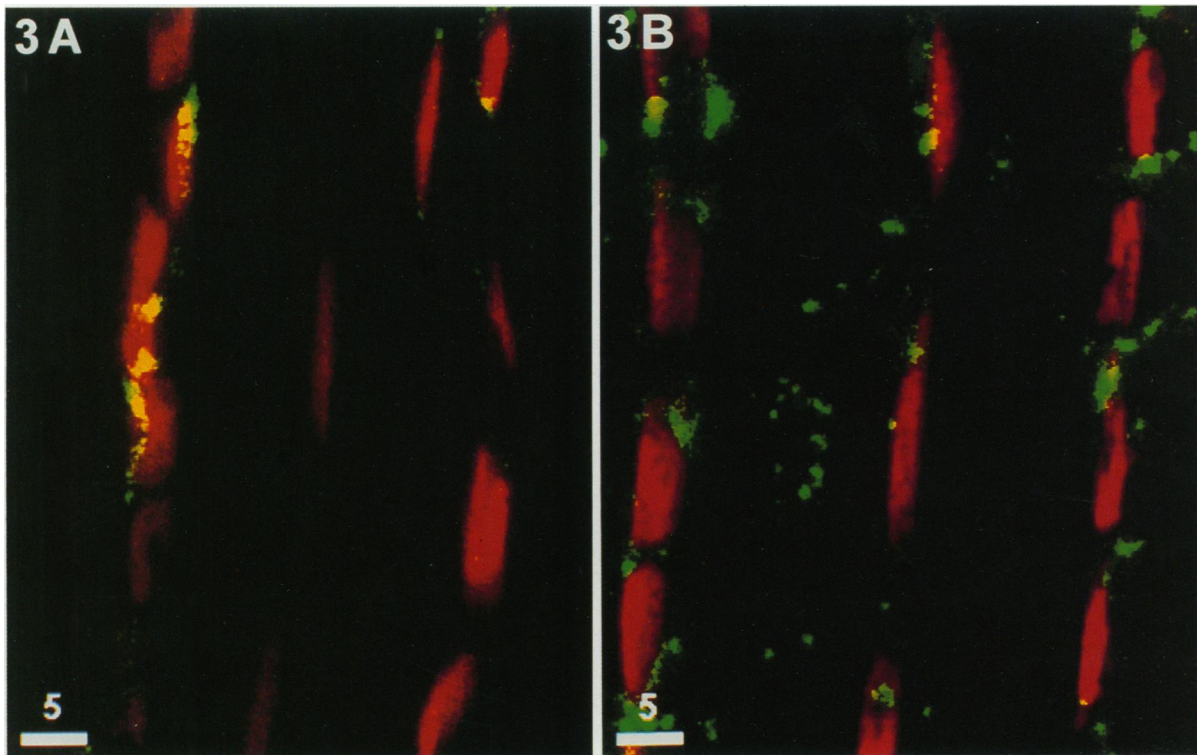


Fig. 3 *A,B*. Direct comparison of immunolabel for cxn32 (*A*) and cxn43 (*B*). Cxn 32 occurs almost exclusively between cells of a row and does not occur on cell processes linking adjacent cell rows. Cxn43 occurs between cells of a row *and* between cells in adjacent rows (arrows).

operation between cells. The intricate 3D network of cell processes associated with collagen bundles is an ideal arrangement for sensing tensile loads transmitted by the tendon—the cell processes must undergo deformation when tendons are stretched, or where they are bent around pulleys and compressed—for the digital flexor tendons, where they pass through the fibrous pulleys associated with the phalanges. Where tendons experience compression they express molecules more typical of cartilage, to form fibrocartilage (Vogel & Koob, 1989; Benjamin & Ralphs, 1995). This fibrocartilaginous differentiation is at its most intense at the site of compression and becomes progressively less further away from the load as the fibrocartilage blends into pure tendon. There is therefore a graded and coordinated response of tendon cells to the mechanical load, just as happens

with calcium transients in the *in vitro* cell deformation experiments (Banes et al. 1995). If this *in vitro* evidence relates to what happens *in vivo*, then it might be expected that frequent calcium transients pass between cells when the tendon is under load. Given the different characteristics of cxn43 and cxn32 junctions and their different distributions between tendons, it could be that the 2 different types of junction are responsible for sensing 2 different forms of loading. For example, one may be important in detecting tensile loads and the other compressive loads. Certainly there is *in vitro* evidence that cxn43 expression is upregulated under conditions of intermittent tensile loading (Banes et al. 1995). The many processes and junctions associated with the cells of the epitenon might suggest that this outer layer (the first to encounter compressive or shear forces)

Fig. 2 *A-F*. Immunofluorescence labelling for connexins in cryosections and wholemounts. Scale bars labelled in μm . (*A*) Cxn43 immunolabel (green) of the central part of a transverse cryosection counterstained with propidium iodide (red; compare with *B*). Lateral cell processes are decorated with bright foci of immunolabel. (*B*) Cxn43 immunolabel of longitudinal cryosection counterstained with DiOC₇ to show cell membranes. Bright foci of immunolabel (green) occur between cell bodies (yellow; large arrows) and on fine longitudinal cell processes (small arrows). (*C*) Cxn43 immunolabel of transverse cryosection at the periphery of the tendon, counterstained with propidium iodide. Bright green foci of label occur between all cells but are particularly prominent between the epitenon (arrow) and first tendon cells proper. Compare with *F*. (*D*) Optical section approximately 28 μm below the surface of a tendon wholemount immunolabelled for cxn43. Bright foci of label are evident and sometimes are seen in longitudinal arrays (arrow), presumably where lateral cell processes of adjacent cells meet. (*E*) Cxn32 immunolabel (green) of transverse cryosection counterstained with propidium iodide (red). Cells label positively, particularly towards the epitenon (e). Label appears closely associated with cells and is not much in evidence between cells (e.g. arrows; compare with *C*). (*F*) High magnification view of a region such as that arrowed in *E* in longitudinal section labelled for cxn32 and counterstained with propidium iodide. The immunolabel can be seen as patches of small foci on the oblique interface between cells within a cell row. At lower magnification the foci cannot be resolved and appear as plaques or large spots of label.

coordinates the response of the internal tendon fibroblasts to load.

A range of other molecules can pass through gap junctions, leading to the possibility of metabolic cooperation between cells. This has been suggested in the region of the myotendinous junction, on the grounds that tendons are relatively poorly vascularised (Tangi et al. 1995). However, it should be noted that signalling networks of gap junctions occur in well vascularised tissues (e.g. heart and smooth muscle, liver, kidney, ciliated epithelia). Thus whilst a metabolic role in tendons cannot be excluded, a sensing, signalling and coordinating role seems more likely.

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